HIGHER ALLELES OF APOLIPOPROTEIN B GENE 3' VNTR: RISK FOR GALLSTONE DISEASE

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ABSTRACT

Background: Imbalance in cholesterol homeostasis may lead to gallstone disease. Apolipoprotein B is sole component of low-density lipoprotein and plays an important role in cholesterol metabolism. The present study was carried out to explore the association of APOB 3' VNTR, exon 26 XbaI and signal peptide insertion/deletion polymorphisms with gallstone disease. 214 ultrasonographically proven gallstone patients and 322 healthy, age and sex matched controls were taken for the study. Genotyping was done using PCR followed by polyacrylamide gel electrophoresis for VNTR and insertion/deletion analysis. For APOB XbaI polymorphism PCR product was digested with XbaI restriction enzyme, followed by agarose gel electrophoresis. All statistical analyses were done using SPSS v11.5. Higher repeat alleles of APOB 3' VNTR polymorphism were more frequent in gallstone patients than in controls. Alleles with more than 57 repeats were present only in patient group. Long (L) alleles with repeat higher than 49, were significantly higher ($P=0.000$; $OR=3.705$, 95% CI 2.577-5.326) and medium (M) alleles were lower ($P=0.000$; $OR=0.406$, 95% CI 0.304-0.542) in patients than in controls. To nullify the effect of gender, data was further stratified into male and female population. APOB 3' VNTR, L alleles were imposing risk and M alleles were protective in both male and female population. APOB XbaI and insertion/deletion polymorphisms were not found to be associated with the gallstone disease. Longer alleles of APOB 3' VNTR occur more frequently in gallstone patients, and may be an important risk factor for the development of gallstone disease. APOB XbaI and signal peptide insertion/deletion polymorphisms may not be contributing to the risk for gallstone disease.

KEY WORDS

APOB 3' VNTR, APOB XbaI polymorphism, APOB insertion/deletion polymorphism, Gallstone disease.

INTRODUCTION

Gallstone disease is a multifactorial disorder where both environmental and genetic factors contribute towards susceptibility to disease. The pathophysiology of cholesterol gallstone formation is complex because a large number of genes regulate cholesterol homeostasis. A prerequisite for the development of cholesterol gallstone is lithogenic bile, which is often the result of enhanced cholesterol synthesis or a

reduced bile acid pool size, or both. Any imbalance in cholesterol homeostasis may lead to cholesterol supersaturation in bile. Lipoproteins, which are transporting vehicles of cholesterol, play an important role in cholesterol homeostasis.

Apolipoprotein B (Apo B) is the main protein component of low-density-lipoprotein (LDL) and contains several domains (1). The expression of apo B gene, present on chromosome 2, is subject to tissue specific regulation (2,3). The human APOB is 43 kb in length with 81 bp signal sequence. Numerous polymorphisms have been identified on this gene. APOB 3' variable number of tandem repeat (VNTR) is located at 72 bp downstream to second putative polyadenylation signal and exhibits a variable number of 11-16 bp repeat (average variation 15 bp) consisting of the adenine-(A) and thymine-(T) rich sequences (4). Association between APOB 3' VNTR

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polymorphism and the level of plasma lipid or lipoprotein or coronary heart disease have been reported in several populations. Higher alleles of *APOB* 3'-hypervariable region (HVR), in particular 48-repeat allele, have been associated with an increased risk of coronary heart disease (5). This *APOB* 3' VNTR region has also been associated with other diseases i.e. Obesity and Hypertension (6,7).

The *APOB XbaI* polymorphism is the result of a single base alteration in the exon 26, which does not change the amino acid sequence of apo B. This polymorphism has been found to be associated with interindividual variability of lipid levels but the results are conflicting (8). The association of *XbaI* polymorphism with coronary heart diseases, non-insulin dependent diabetes mellitus, and cholesterosis has been shown to be associated with dyslipidemia (9,10,11). This polymorphism of *APOB* has also been studied in gallstone patients. Han et al. (12) found X⁺ allele to be significantly higher, but in the Finnish population this polymorphism was not found to be associated with the gallstone disease (11,12).

The *APOB* insertion/deletion polymorphism arises due to an insertion or a deletion of 9 base pairs, which produces a difference of 3 amino acids in the signal peptide (13,14). *In vitro* expression studies have shown that the deletion variant mediates inefficient translocation of apo B protein into the endoplasmic reticulum relative to the insertion variant (15). Variations in plasma lipid concentration have been found associated with this polymorphism (16).

Till now there is no study about the association of gallstone disease with *APOB* 3' VNTR, signal peptide insertion/deletion and exon 26 *XbaI* polymorphisms in Indian population. Moreover, studies evaluating *APOB XbaI* polymorphism in other populations have reported inconsistent results, possibly because of the lack of adequate statistical power, selection bias, population diversity, or genetic admixture. Therefore, aim of the present study was to explore the association of *APOB* 3' VNTR, *XbaI*, and insertion/deletion polymorphisms with gallstone disease. Since the gallstone disease is a worldwide problem and causes social and economic burden on the society, studies on environmental and genetic factors are important to unravel etiopathogenesis of the disease. Present study is also an effort in that direction.

MATERIALS AND METHODS

Subjects : Study comprised of 214 gallstone patients (mean age 44.71±13.20 yr) and 322 controls (mean age 43.98±11.46 yr). Difference in mean age, male/female ratio (69/145 vs.

116/206) and body mass index (22.94±3.90 vs. 23.13±3.85 Kg/m²), between patients and controls respectively were statistically insignificant. All subjects were from North India. The gallstone patients were recruited among inpatients undergoing cholecystectomy and outpatients attending the clinics of Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences. Initially 350 controls were recruited from the healthy staff members of institute and general population of the region and 322 age and sex matched subjects who were found to be negative for gallstone (by ultrasound), diabetes mellitus, coronary artery disease, obesity, and other chronic debilitating disease, were included in the study. All patients and controls were unrelated to each other. Study was approved by local ethical committee of the institute. After an informed consent, blood was taken in EDTA for analysis of DNA. The genomic DNA was extracted from peripheral blood leucocytes pellet using the standard salting out method (17).

Genotyping : For *APOB* 3'VNTR analysis genomic DNA was amplified using primer sequence described by Boerwinkle et al. (18) The PCR product was resolved on 10% polyacrylamide gel electrophoresis along with a 50 bp molecular weight marker (Bangalore Genei, India). The molecular weight of each band was determined using an AlphaMager 1220 v5.5 (AlphaMager™ 1220, Alpha Innotech Corporation, USA) software program where the unknown samples were compared with a 50 bp ladder. The alleles were designated according to their respective base pair size taking the core repeat sequence as 15 bp for *APOB* 3' VNTR.

APOB XbaI polymorphism analysis was done as described previously (12). Allele was designated X⁺ when restriction site for *XbaI* was present. Alternative allele was designated X⁻. For *APOB* insertion/deletion polymorphism, specific region was amplified using primers described by Boerwinkle et al (19). For genotyping PCR products were run on 20% polyacrylamide gel.

Statistical Evaluation : To examine whether the genotype frequencies were in Hardy-Weinberg equilibrium Goodness of fit χ^2 test was used. All other analyses were done using SPSS v11.5 in whole study population and, in male and female population separately. Genotype and allele frequencies were determined by direct counting and compared by χ^2 test or Fisher's exact test. Logistic regression analysis was used to find out contribution of genetic polymorphisms to the risk of disease. Models were constructed to obtain odds ratios for each allele after adjustment for the effect of age, sex, and body mass index (BMI).

RESULTS

Allele and Genotype Frequency

APOB 3' VNTR polymorphism : In control population total 27 alleles, ranging from 30 repeat allele to 57 repeat allele, were observed. In patients total 33 alleles, ranging from 31 repeat allele to 66 repeat allele were observed. Longer alleles (more than 49 repeat allele) were more frequent in patients than in controls, alleles longer than 57 repeat were present only in patient group. The VNTR polymorphism was not in Hardy-Weinberg equilibrium ($P=0.000$) that might be due to presence of a large number of alleles in the studied population.

To make analysis uncomplicated, alleles were coded in three allele model. Up to 38 repeat were kept in short (S) allele group. In medium (M) allele group most common alleles (39-49 repeat) and in long (L) allele group, more than 49 repeats were included. This polymorphism was found to be significantly associated with gallstone disease (Table 1). Longer allele group was imposing significantly high risk ($P=1.52 \times 10^{-13}$; OR=3.705, 95% CI 2.577-5.326) for the disease, on the other hand medium allele group was protective ($P=5.07 \times 10^{-10}$; OR=0.406, 95% CI 0.304-0.542) for the disease.

To nullify the effect of gender, data was further stratified into male and female population. APOB 3' VNTR, L alleles were imposing risk and M alleles were protective in both male and female population (Table 1).

APOB XbaI polymorphism : Studied control and patient population were in Hardy-Weinberg equilibrium ($P=0.532$ and $P=0.151$ respectively) for APOB XbaI polymorphism. In controls heterozygosity was 39.69% and homozygous $X^{+/-}$ was present in 5%.

Frequency of APOB XbaI genotypes has been shown in Table 2. Although frequency of $X^{+/-}$ was lower (2.91% vs. 5.00%) in patients than in controls but difference was not statistically

significant. When stratified gender wise, analysis in male showed slightly higher (57.81% vs. 53.91%) frequency of $X^{-/-}$ and slightly lower frequency of $X^{+/-}$ and $X^{+/+}$ in patients than in controls. Distribution of X^{+} and X^{-} allele was also not different between patient and control groups (Table 3). Separate analysis of male and female population did not reveal any statistically significant difference in frequency of alleles.

APOB insertion/ deletion polymorphism : This polymorphism was in Hardy-Weinberg equilibrium in both patient ($P=0.895$) and control ($P=0.461$) study population.

Table 2 shows the frequency of APOB insertion/deletion polymorphism, genotypes in patients and control group. In patients, frequency of II was higher (71.50% vs. 64.71%), conversely frequency of ID was lower (25.60% vs. 32.94%), than in controls but the differences were not statistically significant. Genotype DD showed almost similar frequency in both the groups.

Gender based stratification of the study population revealed that APOB insertion/deletion polymorphism was associated neither in the males nor in females, with gallstone disease (Table 2). Trends for the distribution of genotypes in males and females were same as observed in total population.

Frequency of D allele was lower and frequency of I allele was higher in patients than in controls (Table 3). Stratification of study population in male and female showed same trends of frequency distribution in patients and controls but no difference was statistically significant.

Risk Assessment : To determine the contribution of different alleles to the risk of gallstone disease logistic regression analysis was used. Results of logistic regression analysis are given in Table 4. APOB VNTR, L alleles were imposing significantly high risk for the gallstone disease. A significant number (6.8%) of gallstone cases could be predicted by L

Table 1 : APOB 3' VNTR polymorphism allele frequencies according to three allele model[#] in gallstone patients and controls

Allele	Total				Male				Female			
	Patient	Control	P-value	OR (95% CI)	Patient	Control	P-value	OR (95% CI)	Patient	Control	P-value	OR (95% CI)
	(No.*=400) N (%)	(No.*=630) N (%)			(No.*=132) N (%)	(No.*=222) N (%)			(No.*=268) N (%)	(No.*=408) N (%)		
S	41 (10.25)	62 (9.84)	0.831	1.046 (0.690-1.586)	12 (9.09)	29 (13.06)	0.259	0.666 (0.327-1.354)	29 (10.82)	33 (8.09)	0.229	1.379 (0.816-2.330)
M	259 (64.75)	516 (81.90)	0.000	0.406 (0.304-0.542)	83 (62.88)	175 (78.83)	0.001	0.455 (0.282-0.734)	176 (65.67)	341 (83.58)	0.000	0.376 (0.261-0.541)
L	100 (25.00)	52 (8.25)	0.000	3.705 (2.577-5.326)	37 (28.03)	18 (8.11)	0.000	4.414 (2.390-8.153)	63 (23.51)	34 (8.33)	0.000	3.380 (2.154-5.304)

[#]S allele= lowest- 38 repeat; M allele= 39-49 repeat; L allele= 50-above repeat; *Total number of chromosome

Table 2 : *APOB XbaI* and insertion/ deletion polymorphism genotype frequency in gallstone patients and controls after stratification according to gender

Genotype	Total			Male			Female		
	Patient N (%)	Control N (%)	P-value	Patient N (%)	Control N (%)	P-value	Patient N (%)	Control N (%)	P-value
<i>APOB XbaI</i> polymorphism									
	No.=206	No.=320		No.=64	No.=115		No.=142	No.=205	
X^{-/-}	117 (56.80)	177 (55.31)	0.738	37 (57.81)	62 (53.91)	0.615	80 (56.34)	115 (56.10)	0.965
X^{+/-}	83 (40.29)	127 (39.69)	0.890	24 (37.50)	46 (40.00)	0.743	59 (41.55)	81 (39.51)	0.704
X^{+/+}	6 (2.91)	16 (5.00)	0.243	3 (4.69)	7 (6.09)	1.000	3 (2.11)	9 (4.39)	0.373
<i>APOB</i> insertion/deletion polymorphism									
	No.=207	No.=255		No.=68	No.=113		No.=139	No.=142	
II	148 (71.50)	165 (64.71)	0.120	46 (67.65)	67 (59.29)	0.261	102 (73.38)	98 (69.01)	0.419
ID	53 (25.60)	84 (32.94)	0.086	20 (29.41)	43 (38.05)	0.237	33 (23.74)	41 (28.87)	0.329
DD	6 (2.90)	6 (2.35)	0.714	2 (2.94)	3 (2.65)	0.909	4 (2.88)	3 (2.11)	0.681

alleles alone, addition of age, sex and BMI in the models led to high increase in R^2 (7.2%); on the other hand, X⁻ allele could explain very few cases (0.7%). *APOB* insertion/deletion polymorphism was also not found to contribute significantly to the risk for gallstone disease even after the adjustment.

DISCUSSION

Gallstone disease is a major problem in India and with privileged circumstances and urbanization in rural villages; its

rate may increase in future. Alterations in lipids have been associated with disease but the mechanism is unknown. Apo B is essential for the synthesis and secretion of both chylomicrons and VLDL (20). Plasma lipoprotein change induced by dietary fat and cholesterol has been correlated to certain *APOB* genetic polymorphisms. Therefore, in present study we analyzed association of three polymorphisms present on *APOB* i.e. 3' untranslated region VNTR, exon 26 *XbaI*, and signal peptide insertion/ deletion, with gallstone disease.

Table 3 : *APOB XbaI* and insertion/deletion polymorphism allele frequency in total subjects and stratified in male and female subjects

Allele	Total			Male			Female		
	Patient N (%)	Control N (%)	P-value	Patient N (%)	Control N (%)	P-value	Patient N (%)	Control N (%)	P-value
<i>APOB XbaI</i> polymorphism									
	No.*=412	No.*=640		No.*=128	No.*=230		No.*=284	No.*=410	
X⁻	317 (76.94)	481 (75.16)	0.509	98 (76.56)	170 (73.91)	0.580	219 (77.11)	311 (75.85)	0.701
X⁺	95 (23.06)	159 (24.84)	0.509	30 (23.44)	60 (26.09)	0.580	65 (22.89)	99 (24.15)	0.701
<i>APOB</i> insertion/deletion polymorphism									
	No.*=414	No.*=510		No.*=136	No.*=226		No.*=278	No.*=284	
I	349 (84.30)	414 (81.18)	0.213	112 (82.35)	177 (78.32)	0.354	237 (85.25)	237 (83.45)	0.557
D	65 (15.70)	96 (18.82)	0.213	24 (17.65)	49 (21.68)	0.354	41 (14.75)	47 (16.55)	0.557

* Total number of chromosome

This is the first study reporting the association between the *APOB* 3' VNTR and gallstone disease. Longer alleles (more than 49) were found to have considerable risk for the development of disease; on the other hand medium allele were protective for the disease in male and female both (Table 1). Regression models revealed L alleles to be strongly associated with the disease and predicted a significant proportion of gallstone disease (Table 4). In other diseases also where lipoprotein metabolism is abnormal, like coronary artery disease and diabetes mellitus, *APOB* VNTR alleles with higher repeat number have been reported to occur more frequently in patients than in controls (21,22,23). Medium alleles are wild type and most likely represent alleles associated with normal expression levels of *APOB* gene and protection for the disease.

Since these repeats are present in 3' untranslated region, they do not play any role in structure of apo B protein. *APOB* 3' VNTR may have some role in gene expression or may be in linkage disequilibrium with other polymorphism either in the same gene or in other nearby genes, whose function is still not known (24, 25). Higher repeat alleles or other alleles with which these alleles are in linkage disequilibrium may have some effect on regulation of gene expression, which ultimately results in abnormal cholesterol transport, supersaturation of bile with cholesterol and development of gallstones. Here we suggest that *APOB* 3' VNTR polymorphism is certainly related to the disease but pathway may not be direct.

Other studied polymorphisms i. e. *APOB* *Xba*I and insertion/deletion, were not found to be associated with gallstone disease in present study. *APOB* *Xba*I, which is due to a single base variation in exon 26 of the *APOB* that does not lead to changes in the amino acid sequence. Studies about this *APOB* *Xba*I polymorphism and gallstone disease have indicated contradictory results. *APOB* *Xba*I polymorphism in Chinese gallstone patients showed that frequencies of $X^{+/-}$ and $X^{-/-}$

were significantly different between the patients and controls and the frequency of X^{+} allele in the patients was significantly higher than that in the controls (0.104 vs. 0.052) (26). Another study in the same Chinese population also showed that frequency of the rare allele X^{+} and the distribution of $X^{+/-}$ genotype were significantly higher in the patient group than controls (27). We did not find any association of *APOB* *Xba*I polymorphism with gallstone disease but frequency of $X^{-/-}$ was slightly higher and frequency of $X^{+/-}$ was slightly lower in patients than controls (Table 2, 3). Trends in our study support the Finish study, in which Juvonen et al (11) found that distribution of *Xba*I genotypes in the gallstone patients did not differ significantly from that of controls.

It appears that *APOB* *Xba*I polymorphism exhibits population specific variation, which may be due to gene and environment interactions. In previous studies number of gallstone patients was also small, which might have influenced the results. *APOB* *Xba*I polymorphism does not lead to changes in the amino acid sequence and can not be implicated at structure level. It is possible that some other polymorphism in its vicinity might be present in Chinese population, which is in linkage disequilibrium with *APOB* *Xba*I polymorphism and showing association with gallstone disease.

APOB insertion-deletion polymorphism alters the N-terminal signal sequence and may alter the apo B secretion and lipoprotein levels (28, 29). Studies have reported that deletion allele carriers may be less responsive to an increased amount of dietary fat (28,30). This is the first study analyzing this association in gallstone disease. In present study gallstone disease was not found to be influenced by this polymorphism. This polymorphism may not be involved in causation of gallstone disease, more studies in different population is needed to confirm the results. In a meta-analysis, this polymorphism was not associated with plasma total, LDL-, or HDL-cholesterol levels (31), which support no role of this polymorphism in dyslipidemia.

Since this is the first study of its kind, more studies in different populations are needed to confirm it. Establishment of high-risk alleles for gallstone development will certainly help in recognition of individuals at high risk and management of disease. Determination of genetic factors will allow lifestyle changes to lower the risk and more intensive medical surveillance to detect the asymptomatic gallstones. Early diagnosis is always very useful to prevent complication associated with gallstone disease, including gallbladder cancer.

Table 4 : Adjusted odds ratios for gallstone disease according to alleles

Polymorphism	Model	P-value	OR	95% CI		R ²
				lower	upper	
APOB XbaI	X ⁺	0.509	1.103	0.825	1.476	0.001
	X ⁻ #	0.502	1.105	0.825	1.480	0.007
APOB VNTR	L ⁺	0.000	3.705	2.577	5.326	0.068
	L ⁻ #	0.000	3.771	2.620	5.429	0.072
APOB I/D	I ⁺	0.214	1.245	0.881	1.759	0.002
	I ⁻ #	0.176	1.297	0.890	1.891	0.010

* Unadjusted for alleles; # Adjusted for age, sex and, BMI

We conclude that longer alleles of *APOB* 3' VNTR occur more frequently in gallstone patients, and may be an important risk factor for the development of disease. *APOB* *Xba*I and signal peptide insertion/deletion polymorphism may not be contributing to the risk for gallstone disease.

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